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Physical, Chemical, Developmental, and Genetic Factors that Modulate the *Agrobacterium-Vitis* Interaction[1]

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ABSTRACT

Tumor formation in *Vitis* species and hybrids, incited by *Agrobacterium tumefaciens*, was altered by chemical, physical, developmental, and genetic variables. Knowledge of the effect of these variables was used to develop a stringent *in vitro* assay system to select parents for a study of genetic factors that modulate tumor formation. Tumor formation was reduced by short day preconditioning of assay plants and by inoculation of the morphological apex of isolated stem segments. Pretreatment of plants with auxin or cytokinin altered specificity in various combinations of strains and host genotypes. All *Vitis* species and hybrids formed tumors in response to strains designated as limited host range, but some displayed a necrotic reaction (cell death at and below site of inoculation) or a null response (same as the response to inoculation with an avirulent strain) to strains designated as wide host range (VC Knauf, CG Panagopoulos, EW Nester [1982] Phytopathology 72: 1545–1549). Screens of F_1 progeny, derived from crosses of null, necrotic, and tumor-producing phenotypes, demonstrated that the null and the necrotic phenotypes were modulated by dominant and recessive host genes. The extent of cellular necrosis in the necrotic phenotype was modified by the morphological location of the inoculation site, by the presence of buds on the host stem, and by deletion of the tryptophane monooxygenase locus gene of the Ti-plasmid.

The tumor-inducing plasmid of *Agrobacterium tumefaciens* contains two regions, termed the virulence (*vir*) and tumor-inducing DNA (T-DNA), that coordinately participate in the excision of DNA from the pathogen and its transfer into the host genome (11, 17). The current model for infection of host cells by *Agrobacterium* predicts that phenolic metabolites produced by wounded plant cells activate plasmid *vir* genes of the bacterium (22). Once activated, the genes modulate the excision and packaging of T-DNA and facilitate its transfer into the host plant genome (17, 22). Expression of T-DNA in host cells alters the endogenous levels of auxin, cytokinin, and ethylene (6, 11, 17), which results in the loss of cellular totipotency (11, 17).

Concepts for *Agrobacterium*-host compatibility have been derived from the analysis of the molecular and genetic correlates of infectivity of several bacterial strains and a diverse array of plant genera. Strains designated WHR[2] infect more than 93 families of plants (3), while strains designated LHR infect only a few families, which include species of *Vitis*, *Rubus*, and a few of *Nicotiana* (1, 3, 17, 27). Bacterial host range and virulence determinants have been attributed to structural and organizational differences in T-DNA loci (1, 17, 21, 27–29, 30) and to structural differences in at least two *vir* loci (30, 31). Some investigators suggest that chromosomal loci might also modulate host selectivity (17, 25). The role of the host genome in *Agrobacterium*-host compatibility has not received as much consideration as that of the pathogen. However, intergeneric (3, 12, 25, 26, 29, 30) and intraspecific and cultivar variations (1, 2, 7–9, 19, 23, 24, 27) in host-response to WHR and/or LHR strains have been reported. A preliminary analysis of *Vitis* hybrids showed that resistance to *A. tumefaciens* was a heritable trait (23).

The primary goal of our research was to develop a system to study the interactions of bacterial virulence genes and host genes so that the molecular and biochemical correlates of *Agrobacterium*-host interaction could be determined.

The first objective was to develop an assay system to identify susceptible and resistant members of a genus in which most species are sexually compatible but display resistance to some strains of the bacterium. The genus *Vitis* was used because it fulfilled the above criteria (23, 24). In this report, we describe the development of an *in vitro* host-pathogen assay and show its utility for the analysis *Agrobacterium*-host compatibility.

MATERIALS AND METHODS

Plant Culture

Aseptic nodal segments obtained from regrowth of dormant canes of various grape species and cultivars were cultured on half-strength MS salts (16) supplemented with 0.09 M sucrose, 0.8% agar, and full strength MS vitamins and organic supplements at pH 5.7. Clonal populations for all species and cultivars were maintained via single node propagation on the same medium. Cultures were maintained under light banks or in growth chambers illuminated by cool-white fluorescent lamps (40 and 50 $\mu mol/m_2/s$, respectively) with a photoperiod

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[2] Abbreviations: WHR, wide host range; LHR, limited host range; NR, necrotic response; *ipt*, isopentenyltransferase locus; *iaa*H, indole-3-acetamide hydroxylase locus; *iaa*M, tyrptophane monooxygenase locus, MS, Murashige-Skoog.

of 16 h light and 8 h dark at a mean temperature of 27°C. Nodal cuttings (for examination of growth regulator effects on pathogenicity) were cultured on MS medium supplemented with either indoleacetic acid (0.1 mg/L) and/or benzyladenine (0.2 mg/L) for 30 d before the assay.

Bacterial Strains

Virulent, avirulent, and mutant strains of *Agrobacterium tumefaciens* (Table I) were maintained on 1.6% agar nutrient broth plates with appropriate antibiotics to maintain selection pressure for particular mutants, in darkness at 28°C. Single colonies were selected from plates and incubated in liquid nutrient broth on a roller drum apparatus (5–7 rpm) for 20 to 24 h before the assay.

In Vitro Host-Pathogen Compatibility Assay

Nodal segments, each containing a single bud, were inserted apical end down (except where noted) into vials containing hormone-free MS medium. Basal ends were inoculated by wire loop with either virulent or avirulent strains or with a broth control. Test samples were maintained under temperature/light conditions as described above. Assays for each host-pathogen pair consisted of five samples and each was replicated three times. Host-pathogen pairs that failed to form tumors were assayed on at least three additional occasions to confirm the negative response.

Delineation of the Host-Pathogen Interaction

Host-pathogen compatibility was determined by scoring the frequency and specificity of tumor induction. The frequency of tumor formation represents the number of tumors formed per number of segments inoculated × 100. A change in host-pathogen specificity was recorded when the frequency of tumorigenesis was reversed by an experimental variable for specific host-pathogen combinations. For example, a change in host specificity would be recorded if a host with a WHR^-, LHR^+ phenotype, to a specific strain of the bacterium, became WHR^+, LHR^- to the same strains, after exposure to a chemical or a physical variable.

We define resistance (scored here as 0) as incompatibility in all samples of a given host-pathogen pair. Incompatibility was manifest by the absence of macroscopically visible cell proliferation at the wound site (similar to inoculation with the T-DNA minus strain, A136) or as a NR[3] that was characterized by progressive collapse and blackening of stem tissue. A host-pathogen pair was considered compatible (scored as T) if one or more assay samples resulted in a tumor.

Table I. *Agrobacterium tumefaciens Strains Used in Host-Pathogen Assays*

All of the WHR strains (except Ag176) were transformants of A136. These synthetic strains were isogenic with respect to bacterial chromosome but contained Ti plasmids from various WHR and LHR strains. Both LHR strains were assayed as wild type and as A136 transformants. No difference in host specificity or tumorigenesis was observed between wild-type and synthetic strains. Strains A328, A338, and A393 contained Tn inserts in various T-region oncogenes. See Figure 6 for details.

Host Range	Strain	Chromosome	Ti plasmid	Source
Avirulent	A136	C58	None	28
Wide	A277	C58	$pTiB_6806$	28
	A348	C58	pTiA6NC	26
	A328	C58	pTiA6NC	29
	A338	C58	pTiA6NC	29
	A393	C58	pTiA6NC	29
	A857	C58	pTi83	12
	A858	C58	pTi86	12
	Ag176	Ag176	Ag176	12
	A281	C58	pTiBo542	21
Limited	Ag63	Ag63	pTiAt63	12
	Ag162	Ag162	pTiAt162	12
	A854	C58	pTiAg63	12
	A856	C58	pTiAg162	26

Criteria for Tumor Induction

Callus formation at the inoculation site was considered to be a tumor if it: (a) survived at least three passages on hormone-free, MS medium supplemented with 1 mg/mL carbenicillin; (b) survived at least one passage on antibiotic- and hormone-free MS medium; and (c) produced opines when cultured on arginine-rich medium. Opine production within putative tumor cell lines and controls was determined after overnight culture of cells in hormone-free, liquid MS medium with or without 50 mM arginine. Negative controls consisted of grape callus cultures from noninfected cultivars cultured on MS medium supplemented with 0.5 mg/L NAA, 0.1 mg/L BA, and 1.0 mg/L GA_3. Positive controls consisted of *Nicotiana tabacum* tumor cultures incited by strain A277. Opines were extracted and assayed according to the procedure of Otten and Schilperoort (18).

It was necessary to verify transformation of tumor lines by dot blot analysis, because all but one of the putative grape tumor lines (that satisfied criteria a and b above) were opine negative (14). DNA was extracted from randomly selected putative tumor lines via a miniprep procedure (4) and blotted onto nitrocellulose paper. LHR^+ tumor DNA was probed with a nick-translated 1.3 kb *Eco*RI fragment and a 0.5 kb *Bam*HI fragment of LHR T-DNA (14). WHR^+ tumor DNA was probed with the nick-translated plasmids pNW 31C-8,29–1 and pNW 31C-2,19–1, which together represent the entire T-region of the WHR T-DNA (14).

RESULTS

To develop a system for the study of bacterial gene-host gene interaction, we determined the effect of various morphological, environmental, and physiological variables on tumorigenesis and host specificity. These studies were necessary to standardize the conditions for the culture of host plants and to identify assay variables that might influence the assay system.

[3] The necrotic response cited in this manuscript is equivalent to the hypersensitive response described in Yanofsky *et al.* (30).

Table II. *Effect of Inoculation Location on the Frequency of Tumor Development on Isolated Stem Segments of Vitis Species Hybrid Seyval*

Numbers represent the percentages of stems (n = 15) that displayed the tumor phenotype.

Bacterial Strain	Host Range	Inoculation Position	
		Apical	Basal
A136	Avirulent	0	0
A277	Wide	20	75
Ag176	Wide	60	100
A854	Limited	40	60

Effect of Stem Polarity

Single node stem segments were inoculated on either the apical or basal cut surface to determine the effect of morphological location on tumor formation. The basal cut surface produced a greater number of tumors than the apical cut surface (Table II). However, position of the inoculation site did not alter host specificity, because tumors formed with both WHR$^+$ and LHR$^+$ strains.

Effect of Photoperiod Preconditioning

To determine photoperiod effects on growth, tumor frequency, and host specificity, we assayed nodes from 'Seyval' and 'Steuben' mother plants exposed to 16-h or 8-h light periods. Exposure to short days had no significant effect on node number or shoot length (14). The frequency of tumor formations, however, was reduced by short-day treatment for both cultivars, but specificity to WHR or LHR strains was unaltered (Table III). Both cultivars formed tumors in response to the LHR strain, but only Seyval formed tumors in response to the WHR strain.

Effect of Growth Regulator Pretreatment

The growth of some grape cultivars or species was relatively slow on media lacking growth regulators (14). For example, bud elongation on some cultivars either was not influenced by auxin ('Cabernet Franc'), was reduced by auxin ('Grenache'), or was increased by the addition of auxin (*V. lincecummi*) (Fig. 1). In the extreme example, buds on nodal segments from 'Aramon' and 'Jaeger 70' would not elongate unless auxin was added to the medium (data not shown, see ref. 14). Therefore, to obtain shoots for analysis, we sometimes found it necessary to supplement the medium with growth regulators.

Table III. *Effect of Photoperiod Preconditioning on Host Response to Wide and Limited Host-Range Strains of Agrobacterium*

Cultivar	Daylength	Tumors[a]		
		A136	A277	A854
			%	
Seyval	16	0	86	85
	8	0	40	43
Steuben	16	0	0	87
	8	0	0	41

[a] Bacterial strains were A136, avirulent; A277, WHR; A854, LHR.

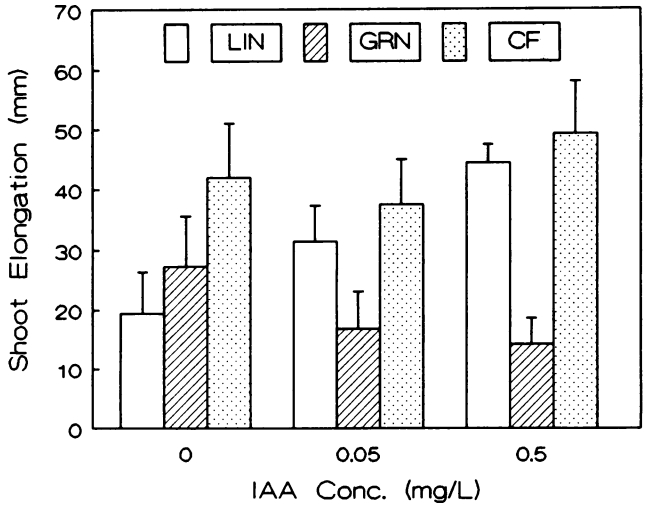

Figure 1. Effect of IAA on *in vitro* bud elongation of grape species and cultivars. LIN, *V. lincecummi*; GRN, *V. vinifera* Grenache; CF, *V. vinifera* Cabernet Franc. Error bars represent the standard error of the mean for 10 samples per treatment. Bud elongation on single node explants (without leaves initially) was determined 30 d after initiation of culture.

Others have reported that growth regulators influence tumor formation (10, 11). It was necessary, therefore, to determine the effect that growth regulator added to shoot production medium had on the subsequent host-pathogen interaction. To determine if growth regulator application altered the specificity of host-pathogen compatibility, we cultured explants on medium containing either auxin or cytokinin or both. After 30 to 40 d exposure to growth regulators, nodal segments from treated and control plants were assayed as described above. Pretreatment with growth regulators resulted in altered patterns of bud elongation (data not shown, see ref. 14) and host specificity (Table IV).

Explants from Steuben (WHR$^-$, LHR$^+$ phenotype) assumed the WHR$^+$, LHR$^+$ phenotype when pretreated with both auxin and cytokinin but not when either growth regulator was used alone. In contrast, the cultivar 'Barrett' (WHR$^+$, LHR$^+$) became WHR$^+$, LHR$^-$ in response to all growth regulator pretreatments. The specificity of the cultivar Seyval (WHR$^+$, LHR$^+$) was not altered by pretreatment with growth regulators. Explants from *V. lincecummi* and 'Concord' (both WHR$^-$, LHR$^+$) displayed complete reversals in phenotype (WHR$^+$, LHR$^-$) when mother plants were pretreated with auxin.

Screen of *Vitis* Germplasm for *Agrobacterium* Compatibility

Six species and 31 cultivars and interspecific hybrids were assayed for tumorigenesis following inoculation with five WHR and four LHR strains of *A. tumefaciens* via the *in vitro* method. All nodal explants were derived from mother plants

Table IV. *Effect of Growth Regulator Preconditioning on the Host Response to Wide and Limited Host Range Strains of Agrobacterium*

Mother plants were cultured on media supplemented with either or both IAA, IBA, or BA.

Cultivar	Pretreatment	Bacterial Strain[a]	
		W	L
V. labruscana Steuben	None	0	T
	IAA	0	T
	BA	0	T
	IAA + BA	T	T
V. rupestris Barrett	None	T	T
	IAA	T	0
	BA	T	0
	IAA + BA	T	0
Vitis hybrid Seyval	None	T	T
	IAA	T	T
	BA	T	T
	IAA + BA	T	T
V. lincecummi	None	0	T
	IAA	T	0
V. labruscana Concord	None	0	T
	IBA	T	0

[a] Nodes isolated from mother plants were inoculated with either avirulent A136, wide host range A277 (W), or limited host range Ag162 (L) strains and scored for tumors (T) or no tumors (O).

cultured *in vitro* on growth regulator-free medium except for the cultivars Aramon and Jaeger 70, which required auxin for bud elongation. All mother plants were grown under 16-h daylength and all segments were inoculated at the morphological base.

All of the grape species, cultivars, and species hybrids formed tumors in response to the LHR strains. The following species, cultivars, and species hybrids formed tumors in response to all of the WHR strains: *V. vinifera* (Alicante Bouschet, Aramon, Cabernet Franc, Cabernet Sauvignon, Chardonnay, Grenache, Ribier, Riesling); *V. rupestris* (Barrett, Ganzin); *V. labruscana* (Alba, Niagra, Golden Muscat); and *Vitis* species hybrids (NY66.717.4, Seyval, Seibel 4986, Siebel 5898, Siebel 14.596, Oberlin, Noah, Marachel Foch, Baco Noir). Incompatible reactions to some of the WHR strains were observed (Table V). The *V. labruscana* cultivars (Concord, Ontario, and Steuben), the species *V. lincecummi* and the species hybrids (Aramon × *rupestris*, Ganzin No. 1, Catawba, and NY65.467.8) did not form tumors in response to the WHR strains A277 and A348. Of the species and cultivars examined, only *V. lincecummi* displayed a WHR phenotype to more than two of the five WHR strains.

A grape line was considered compatible with an *Agrobacterium* strain if it retained hormone autonomous growth for three passages on hormone and antibiotic-free medium and produced opines. Cell lines that were hormone autonomous but did not produce opines were considered transformed only if the DNA isolated from them contained sequences homologous to the T-region of the inciting organism. However, only one tumor line, 'Seyval'-A277, produced opines (14). Therefore, to prove transformation, DNA from randomly selected, putative tumor lines was subjected to dot blot analysis and probed with nick-translated DNA from the T-regions of the inciting organism (Fig. 2). DNA from a tobacco tumor line which contained one copy of T-DNA per genome was used as a reference to estimate the number of T-DNA copies in the grape genome assuming that the grape and tobacco genomes are of equivalent size. Row 1 of columns A, B, C, and D represent 0.5, 5.0, 2.0, and 1.0 copy reconstructions of tobacco tumor DNA. None of the grape or tobacco callus controls contained DNA that hyridized with the WHR T-DNA probe (A5, A6, A7, A11, B4, C6, D4, and D7). All of the putative tumor lines of grape and tobacco, with the exception of the Steuben hybrid No. 19 (A10) and some of the Seyval tumor lines (B12, C9, C10, D10, 11, and 12), hybridized to the WHR T-DNA probe. Two of the four independent Seyval tumors incited by A176 did not hybridize to the probe, but two (C11, 12) displayed very faint hybridization to the probe. Seyval tumors incited by A277 (D10, 11, 12) and A857 (B12) did not hybridize to the probe. However, two of the Seyval tumor lines incited by A277 (D8, 9) did hybridize with the probe.

DNA from grape tumor lines incited by LHR strains was probed with bacterial DNA that represented the t-A region

Table V. *Tumorigenesis of Vitis Species and Hybrids in Response to WHR and LHR Strains of Agrobacterium tumefaciens*

None of the species and cultivars responded to the avirulent strain A136.

Species Cultivar	WHR[a]					LHR[a]			
	A277	A348	A857	A858	Ag176	Ag63	Ag162	A854	A856
V. labruscana									
Concord	0	0	T	T	T	T	T	T	T
Steuben	0	0	T	T	T	T	T	T	T
Ontario	0	0	T	T	T	T	T	T	T
V. lincecummi	0	0	0	0	T	T	T	T	T
Vitis species hybrids									
Aramon × rupestris	0	0	T	T	T	T	T	T	T
Ganzin No. 1	0	0	T	T	T	T	T	T	T
Catawba	0	0	T	T	T	T	T	T	T
NY65.467.8	0	0	T	T	T	T	T	T	T

[a] T, tumor; 0, no tumor.

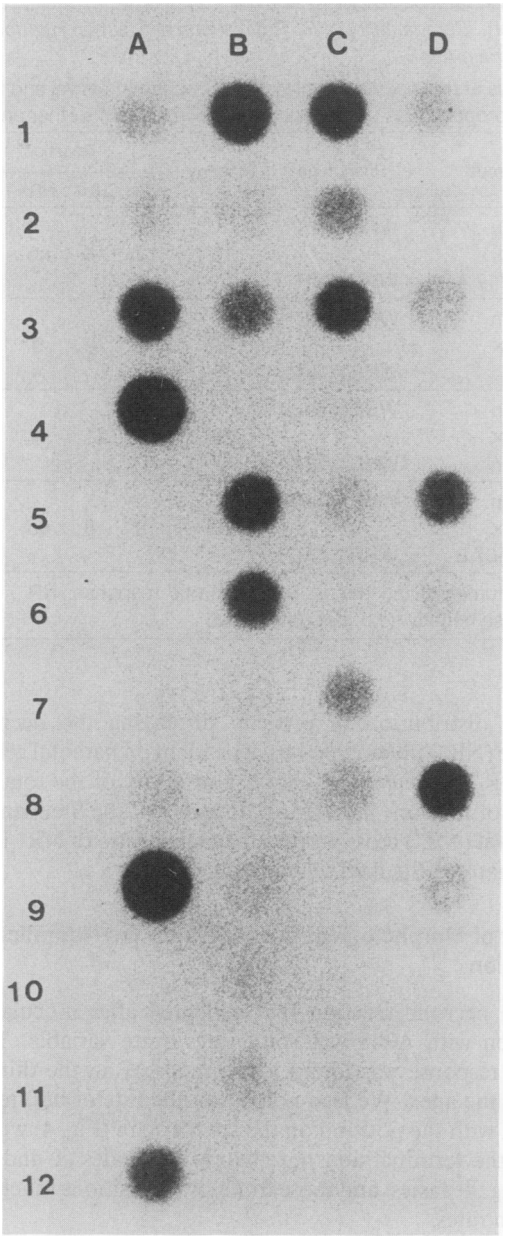

Figure 2. Hybridization of DNA from grape and tobacco tumors with probes representing the entire T-DNA of the WHR strain A348. Plant name is preceeded by bacterial strain. Letters after bacterial strain represent identity of an independently derived tumor line. Each dot represents an equivalent amount of DNA from tumor or callus controls. Column A: row 1, 0.5 copy reconstruction; row 2, Seyval-Ag63c; row 3, *N. tabacum* John Williams-A277a; row 4, *N. tabacum* × *N. glauca*-A277a; row 5, *N. glauca* callus; row 6, *Vitis* hybrid Seyval callus; row 7, *N. tabacum* Havana-callus; row 8, *N. tabacum* John Williams-A348h; row 9, *N. tabacum* E9-A277 (18 copies of T-DNA/genome); row 10, *V. labruscana* Steuben × NY65.467.8 No. 19-A277a; row 11, *V. labruscana* Steuben × NY66.717.4 No. 138-callus; row 12, *V. labruscana* Steuben × NY66.717.4 No. 127-A277b. Column B: row 1, 5.0 copy reconstruction; row 2, *V. labruscana* Steuben-R1b; row 3, *Vitis* hybrid Seyval-R1d; row 4, Steuben-callus; row 5, *V. vinifera* Aramon × *V. rupestris*-857b; row 6, Steuben × NY65.467.8 No. 14-A857a; row 7, *V. rupestris*-A857b; row 8, *V. rupestris*-A857b; row 9, *V. labruscana* Concord-857a; row 10, Concord-A857b; row 11, Steuben-A857a; row 12, Seyval-A857a. Column C: row 1, 2.0 copy reconstruction; row 2, *N. cavicola*-A277b; row 3, *N. cavicola*-A277b; row 4, *N. cavicola*-A176b; row 5, *N. cavicola*-A176a; row 6, *N. cavicola*-callus; row 7, *N. tabacum* × *N. glauca*-A176b; row 8, *N. tabacum* × *N. glauca*-A176a; row 9, Seyval-A176i; row 10, Seyval-A176h; row 11, Seyval-A176f; row 12, Seyval-A176a. Column D: row 1, 1.0 copy reconstruction; row 2, *V. vinifera* Riesling-A277a; row 3, *V. vinifera* Cabernet Sauvignon-A277e; row 4, *V. vinifera* Aramon-callus; row 5, *V. vinifera* Chardonnay-A277b; row 6, *Vitis* species hybrid Marachel Foch-A277e; row 7, Seyval-callus; row 8, Seyval-A2771; row 9, Seyval-A277k; row 10, Seyval-A277a; row 11, Seyval-A277f; row 12, Seyval-A277b.

which contained the isopentenylphosphotransferase (*ipt*) gene and the t-B region (30) which contained the *iaa*M and *iaa*H auxin biosynthesis genes (Fig. 3). Dots on identical positions under t-A and t-B in Figure 3 represent DNA from the same tissue sample. None of the control callus cultures (A1, A2, A6, B6, and B11), except for Concord (A2), hybridized with either of the probes. The Concord control callus (A2) displayed traces of homology to both probes. Only two tumor lines (Siebel 5998, B9 and Steuben × NY66.717.4 No. 127 A12) contained DNA that failed to hybridize to either probe. However, other tumor lines of Siebel, incited by the same bacterium, contained DNA that hybridized with the two probes (B8, 10). DNA from the Steuben tumor line, incited by the WHR strain A277, did not hybridize with either of the LHR probes.

Equal amounts of tumor DNA were blotted on to the filter. Therefore, differences in blot intensity are likely due to differences in copy number of inserted DNA. Six of the tumor lines (A5, A8, A10, B5, B8, B12) contained greater copy numbers of the *ipt* locus (t-A probe) relative to the *iaa*M-*iaa*H loci (t-B probe). Of these lines, only one, Seyval-A856 (B5), showed no hybridization with the t-B region. One Steuben tumor (A7) showed significantly greater hybridization to the t-A than the t-B probe, because the inciting *Agrobacterium* strain, A856d11, contained a deletion of the *iaa*H-*iaa*M loci (30). All of the remaining tumor lines (A3, A4, A9, B1, B2, B3, B4, B7, and B10) contained greater copy numbers of the t-B loci relative to t-A locus. Of these, only (A9) was anticipated because the inciting strain A856d7 contained a deletion of the t-B region (30).

Screen of F_1 Progeny for Host-Pathogen Interaction

The distribution of progeny resistant and susceptible to the WHR strain A348 was determined in four F_1 populations of Steuben hybrids. Strain A348 was chosen because it was incompatible with Steuben and because the incompatible interaction was associated with the induction of a necrotic reaction (29). The phenotypes of the parents and the distribution of resistant and susceptible progeny are shown in Table VI. Steuben was crossed with two susceptible and two resistant parents. In the cross with the susceptible NY66.717.4 cultivar, most of the progeny were susceptible. In contrast, progeny of the cross with another susceptible cultivar, Seyval, resulted in a majority of progeny with a resistant phenotype. When Steuben was crossed with the resistant parent NY65.467.8 or selfed, the segregation ratios favored a higher proportion of resistant progeny (selfed) or an equal ratio of resistant and susceptible progeny (NY65.467.8).

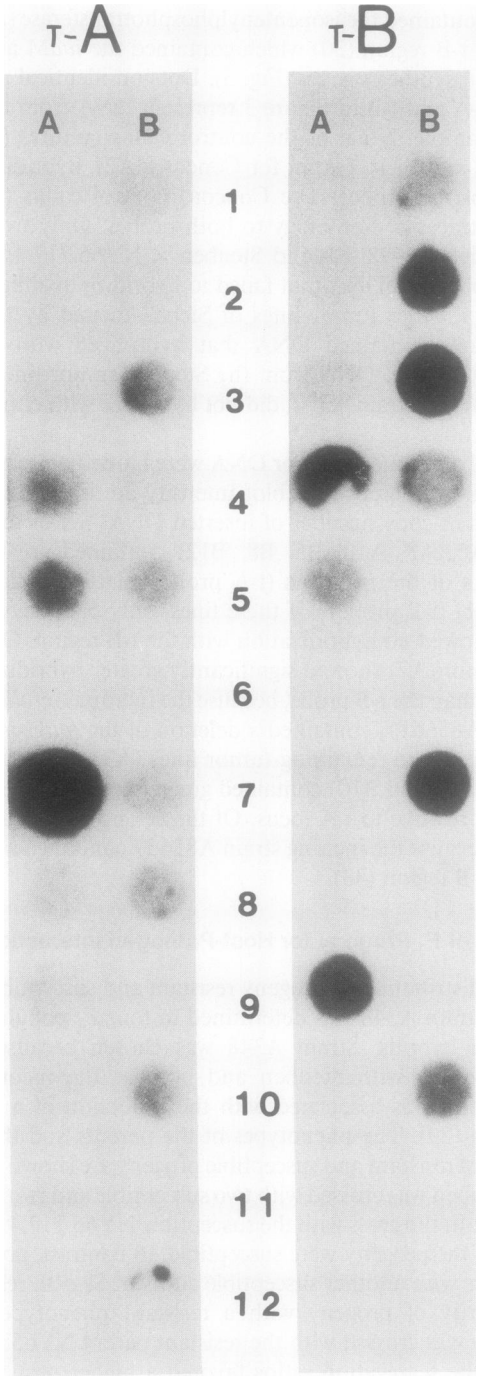

Figure 3. Dot hybridization of *Vitis* tumors incited by LHR strains of *A. tumefaciens*. DNA from callus controls or tumors was bound to nitrocellulose filter and hybridized with probes representing t-A [*ipt* locus] or t-B [*iaa*M-*iaa*H loci] region of an LHR Ti-plasmid. Column A: row 1, *V. vinifera* Chardonnay-callus; row 2, *V. labruscana* Concord-callus; row 3, Concord-Ag63b; row 4, *V. vinifera* Riesling-A854b; row 5, Chardonnay-A854a; row 6, *V. vinifera* Aramon-callus; row 7, *V. labruscana* Steuben-A856d11 [deletion of *iaa*H-*iaa*M in tB-DNA]; row 8, Steuben-A856a; row 9, Steuben-A856d7 [deletion of *ipt* in tB-DNA]; row 10, Steuben × NY65.467.8 No. 34-A854d; row 11, Steuben × NY66.717.4 No. 127-A854a; row 12, Steuben × NY66.717.4 No. 127-A277c. Column B: row 1, *Vitis* species hybrid Seyval-Ag63c; row 2, Seyval-A854-b; row 3, Seyval-A856a; row 4, Seyval-A856b; row 5, Seyval-A856c; row 6, Seyval-callus; row 7, *Vitis* species hybrid Marachel Foch-A854b; row 8, *Vitis* species hybrid Siebel 5898-A854a; row 9, Siebel 5898-A854b; row 10, Siebel 5898-A854c; row 11, Seyval-callus; row 12, *N. tabacum* John Williams × *N. glauca*-Ag63a.

Table VI. *Compatibility of F1 Populations of Steuben Hybrids to the WHR Strain A348*

Callus at the inoculation site of the Steuben × Seyval and Steuben selfed progeny was scored tumor positive without further analysis.

Parent	Phenotype	Progeny	Reaction[a]			
			T	0	NR$^+$	NR$^-$
Steuben × NY66.717.4	WHR$^-$, NR$^+$ / WHR$^+$, NR$^-$	16	12	4	ND	ND
Steuben × Seyval	WHR$^-$, NR$^+$ / WHR$^+$, NR$^-$	30	2	28	9	19
Steuben × Steuben	WHR$^-$, NR$^+$ / WHR$^-$, NR$^+$	30	9	21	4	17
Steuben × NY65.467.8	WHR$^-$, NR$^+$ / WHR$^-$, NR$^-$	16	8	8	ND	ND

[a] T, tumor; 0, no tumor; NR$^+$, necrotic response; NR$^-$, necrotic response negative; ND, not determined.

The distribution of progeny displaying the necrotic-response (NR$^+$) phenotype was dependent on parental genotype, and NR$^+$ progeny were always a minority of the total population of resistant individuals. Progeny of the Steuben (NR$^+$) × Seyval (NR$^-$) cross produced a higher ratio of NR$^+$ individuals than the Steuben selfed population.

Effect of Morphological Correlates on the Necrotic Reaction

The necrotic reaction that appeared after inoculation of Steuben with A348 was sometimes quite variable. The necrotic response was found to be maximal on the third node below the apex. We also noted that the rate of bud regrowth varied with the position on the source stem (Fig. 4) with buds from the terminal and next two lower nodes (1 and 2) displaying the fastest and those from lower positions much slower growth rates.

Effect of Stem and Bud Position on the Necrotic Reaction

The apical or basal ends of isolated segments, in which bud position varied or which did not contain buds, were inoculated with strain A281. The presence of buds on stem segments tended to repress the necrotic response, and the effect appeared to be independent of bud position (Fig. 5).

Effect of Mutations in T-Region Oncogenes on the Induction of Stem Necrosis

The necrotic response of Steuben to A348 was abolished when the *vir*C locus was mutated by transposable elements

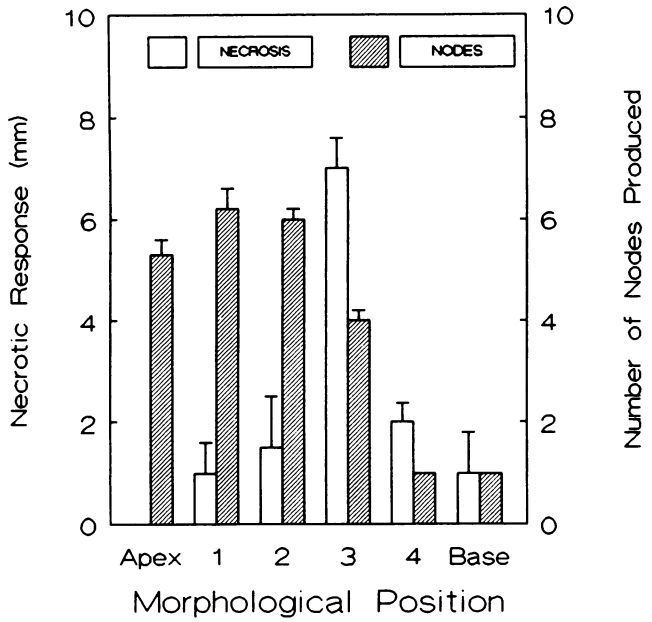

Figure 4. Effect of node position on bud elongation and necrotic reaction to *Agrobacterium* strain A348. Nodes containing a single bud were isolated from various positions of 30-d-old tissue cultured plants. The mean number of nodes produced by each was determined 30 d after culture initiation. The data represent 10 samples for each position, each replicated twice. The extent of necrosis was determined with the aid of a calibrated magnifying ocular and represents the distance, from the inoculation site, that turned brown 21 d after inoculation. Ten samples were used for each inoculation site. Error bars represent the SE of the mean.

(30). We suggested that *vir*C must act in concert with the T-DNA because the *vir* region alone did not induce the necrotic reaction. We examined the effect of transposon-induced mutants (5) in *ipt*, *iaa*M, or *iaa*H loci of A348 to test the hypothesis that T-region oncogenes modulated stem necrosis. Another WHR strain, A281, was also examined because it was chromosomally isogenic to A348 but contained a succinamopine Ti plasmid. It was more virulent on some plant cultivars than the octopine plasmid strain, A348 (2).

Insertion of Tn5 in the *ipt* locus (A338) had no effect on the necrotic response (Fig. 6). Inserts into the *iaa*M locus (A328) appeared to slightly reduce necrosis, whereas an insert into *iaa*H locus (A393) resulted in a significant reduction in the necrotic reaction. Stems inoculated with A281 ranked highest in necrotic reaction but variability was considerable.

DISCUSSION

Our host-pathogen assay system employed clonally propagated shoots to provide genetically identical host replicate samples. Nevertheless, considerable residual variation resulted from developmental or physiological factors and from imposed physical or chemical variables. Factors that contributed to variation in both the frequency and specificity of tumorigenesis in *Agrobacterium*-grape interactions appeared to involve altered growth regulator balance in the host system. For example, the increased frequency of tumorigenesis on the basal ends relative to apical ends of isolated stem segments might reflect an enhanced auxin content resulting from preferential basal movement of endogenous auxin. Similar effects of tissue polarity were observed in carrot (20). Similarly, the reduction of tumorigenesis by short day conditions might reflect altered growth regulator balance, because short days result in cessation of grape stem elongation and the onset of dormancy which also resulted in reduced tumor formation in *Prunus* (15).

The only variable that influenced both frequency and specificity of *Agrobacterium*-grape interaction was pretreatment of assay plants with auxin and/or cytokinin. In general, growth regulator pretreatment resulted in a shift from a null to a tumor-forming phenotype with WHR strains and a reverse shift with the LHR strains (Table IV). These apparent shifts in host specificity were not correlated with the ability of the host plant to respond, via cell elongation, to the applied

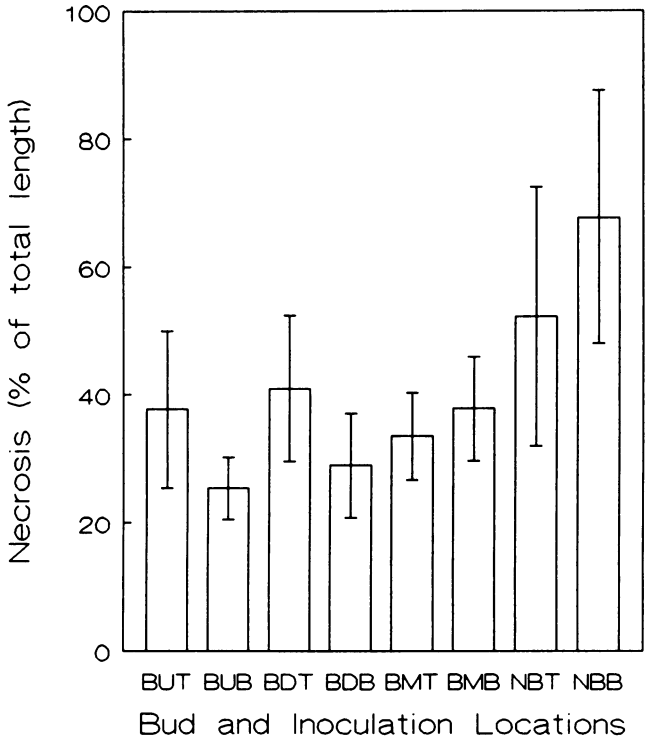

Figure 5. Effect of bud position and bud presence on magnitude of necrosis induced by strain A281. The extent of necrosis represents the ratio of necrotic to nonnecrotic stem tissue 21 d after inoculation with the WHR-agropine strain A281. Necrotic response was determined as noted in Figure 4. BUT, bud at morphologic apex (BU), inoculation site morphologic apex (T); BUB, bud at morphologic apex (BU), inoculation site morphologic base (B); BDT, bud at morphologic base (BD), inoculation at morphologic apex (T); BDB, bud at morphologic base (BD), inoculation at morphologic base (B); BMT, bud in middle of segment (BM), inoculation site at morphologic apex (T); BMB, bud in middle of segment (BM), inoculation site at morphologic base (B); NBT, no bud on segment (NB), inoculation site at morphologic apex (T); NBB, no bud on segment (NB), inoculation site morphologic base (B). Each treatment consisted of five samples and each was replicated twice. Bars represent the standard error of the mean.

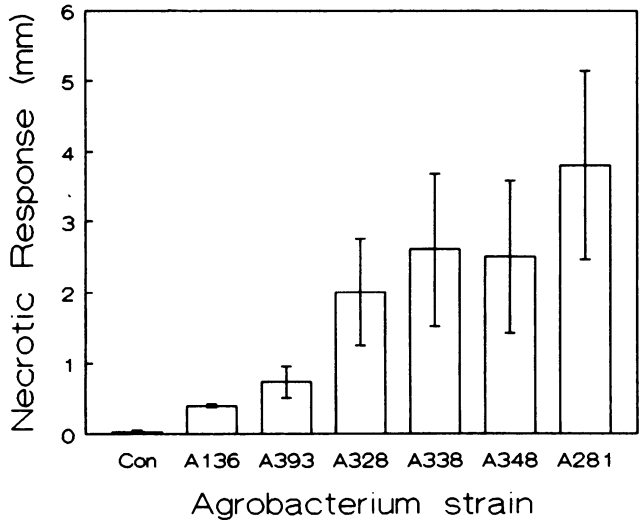

Figure 6. Effect of T-DNA mutants on stem necrosis in the cultivar Steuben. All stems were inoculated on the morphologic base and were obtained from nodes 2, 3, and 4 of *in vitro* cultured mother plants. Con, Uninoculated control; A393, Tn insert in *iaa*H; A338, Tn insert in *ipt*; A328, Tn insert in *iaa*M; A348, wild-type strain; A136, avirulent control. Error bars represent SE of the mean.

growth regulator. For example, Steuben, Concord, and Barrett displayed no change, increased, or decreased growth, respectively, in response to auxin pretreatment (14), but all displayed a shift in specificity to pathogen strains. We suggest that the effects of growth regulators on tumor frequency and host-pathogen specificity are independent of their mechanisms for modulating cell expansion.

We know that host selectivity is modulated, in part, by the Ti-plasmid (1, 17, 29) and that LHR strains differ from WHR strains via a deletion in the promoter region of the LHR *ipt* locus (30) and by structural differences in the *vir*C (30, 31), *vir*A loci (13), and the *vir*A-*vir*B junction (M Zapor, personal communication). We show here that host-pathogen specificity is also modulated by the host genome. For example, the cultivar Steuben was infected by wild-type LHR strains and those that contained only *iaa*M-*iaa*H or only the *ipt* loci (Fig. 3). In contrast, the cultivar Seyval formed tumors in response to the wild-type strain and only those that contained the *ipt* locus (29, 30) (see Fig. 3 which also shows that all Seyval tumors contain homologous sequences to *ipt* but not always to *iaa*M-*iaa*H loci). We suggest that some host plants can functionally complement pathogen oncogene function. An analysis of our Steuben × Seyval hybrid populations may provide insights into the genetic and biochemical nature of host-pathogen oncogene complementation.

The observation that some grape species and cultivars displayed a null or necrotic phenotype to WHR strains but not to LHR strains was surprising. The observation that the LHR strain Ag162, which contains a defective cytokinin synthesis locus (*ipt*) (30), not only was not complemented by additional cytokinin, but in fact produced a null phenotype on the cultivar Barrett when treated with cytokinin (Table IV), suggests that some of the T-region genes (for specific host interactions) might modulate the null and necrotic host phenotypes. Another example of this was the necrotic reaction in Steuben induced by strain A348 (Fig. 6). We showed previously that *vir*C of A348 was required for necrosis (30), and here we report that a mutation in the *iaa*H locus of A348 (strain A393 in Fig. 6) results in a significant decrease in stem necrosis. That both *vir*C and *iaa*H are required for stem necrosis is clear, but how these loci interact is not. One plausible hypothesis is that IAA activity is up-regulated by *vir*C and that excessive auxin activity results in enhanced senescence (by stimulation of ethylene production?). It would be interesting to learn if double mutants of *vir*C and either or both *iaa*H or *iaa*M alter the tumor phenotype in Steuben.

Further evidence to support the concept that modulation of host response phenotypes are controlled by growth regulators are: (a) auxin applications exerted the most pronounced and consistent shifts in *Agrobacterium*-grape specificity (Table IV); (b) the induction of the necrotic reaction by WHR strains was influenced by the morphological position of the inoculation site (Figs. 4 and 5), and the position or presence of a bud relative to the inoculation site (Fig. 5). Factor(s) from buds, independent of their position relative to the inoculation site, repressed the necrotic reaction and maintained polar differences in necrosis when they were asymmetrically positioned with respect to inoculation site. Removal of bud position asymmetry with respect to inoculation site, *i.e.* buds in middle of stem (BMB, BMT in Fig. 5) or no buds on the stem reversed or abolished the polarity of the necrotic reaction. Collectively, these data can be interpreted to suggest that endogenous gradients of substances (perhaps growth regulators with auxin/cytokinin activity) modulate the necrotic reaction and host specificity to various *Agrobacterium* strains. That balances of growth regulator activity modulate both tumor formation and host-pathogen specificity as well as necrotic reactions to *Agrobacterium* indicates that host-gene expression that is dependent on gradients or activities of growth regulators can determine whether host cells express symptoms of susceptibility or resistance.

Host genes that modulate infectivity and host specificity are not known. Our data show that segregation ratios of phenotypes displayed large variations even when parental phenotypes were similar, suggesting a multigene modulation of the infection process. In further support of this hypothesis, our data show that the necrotic phenotype appears to be modulated by recessive genes and the null phenotype by dominant genes. We make this assumption because the F1 progeny, derived from a cross with two NR^+ parents, was composed of a small population of NR^+ individuals and a much larger population of WHR-resistant, but NR^- individuals (Table VI).

Further efforts to characterize the number and function of host genes that modulate infection will provide materials for the dissection of the response phenotypes into their various components. Identification of genetic control points of the resistance reactions should provide materials and a means to sort out the pleiotropic effects of growth regulators and plant wound products on the modulation of *Agrobacterium*-host interaction. The mechanism for growth regulator modulation of host-pathogen specificity remains to be explored. Some potential points of regulation that might be explored are: growth regulator-induced modification of the host cell wall

and subsequent attachment of the bacterium, the effect of growth regulators on quantitative or qualitative changes in plant wound metabolites that regulate induction or repression of bacterial virulence genes, and growth regulator modulation of methylation or demethylation of pathogen-modified host genomes.

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